Herbivore intoxication as a potential primary function of an inducible volatile plant signal

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Running headline: Direct benefits of an induced volatile

Summary

1. Plants release herbivore-induced volatiles (HIPVs), which can be used as cues by plants, herbivores and natural enemies. Theory predicts that HIPVs may initially have evolved because of their direct benefits for the emitter and were subsequently adopted as infochemicals.
2. Here, we investigated potential direct benefits of indole, a major HIPV constituent of many plant species and a key defense priming signal in maize. We used indole deficient maize mutants and synthetic indole at physiologically relevant doses to document the impact of the volatile on the generalist herbivore *Spodoptera littoralis*.
3. Our experiments demonstrate that indole directly decreases food consumption, plant damage and survival of *S. littoralis* caterpillars. Surprisingly, exposure to volatile indole increased caterpillar growth. Furthermore, we show that *S. littoralis* caterpillars and adults consistently avoid indole producing plants in olfactometer experiments, feeding assays, and oviposition trials.
4. *Synthesis.* Together, these results provide a potential evolutionary trajectory by which the release of a HIPV as a direct defence precedes its use as a cue by herbivores and an alert signal by plants. Furthermore, our experiments show that the effects of a plant secondary metabolite on weight gain and food consumption can diverge in a counterintuitive manner, which implies that larval growth can be a poor proxy for herbivore fitness and plant resistance.

Key-words: indole, plant defence, plant herbivore interactions, priming, volatile signaling, plant toxin, herbivore induced plant volatile

Introduction

Upon herbivore attack, plants release herbivore induced plant volatiles (HIPVs) (Heil 2014). HIPVs are used as cues or signals by a wide variety of organisms: Natural enemies of herbivores for instance can use HIPVs as cues to locate their prey (Turlings *et al.* 1990; Amo *et al.* 2013; Sabelis & Dicke 1987). Herbivores can use them to avoid competition (Pallini *et al.*, 1997, De Moraes *et al.* 2001; Sanchez-Hernandez *et al.* 2006) or to aggregate on host plants (Halitschke *et al.* 2008; Carroll *et al.* 2008; von Mérey *et al.* 2013; Robert *et al.* 2012). Plants themselves can perceive HIPVs and employ them to pre-activate their defences (Engelberth *et al.* 2004; Frost *et al.* 2008; Heil & Silva Bueno 2007). For HIPVs to function as so called infochemicals (Dicke & Sabelis 1988), plants must first have come to produce them, and receiver organisms must have acquired the capacity to detect and respond to them. Because parallel evolution of both emission and perception is unlikely (Janssen *et al.* 2002), HIPVs may not have evolved as infochemicals initially. The ambivalent biological impacts of HIPVs on plant associated organisms (Dicke & Baldwin 2010; Poelman *et al.* 2012) and the absence of conclusive evidence regarding their benefits as tritrophic signals (Schuman *et al.* 2012; Heil 2014) adds to the current uncertainty regarding the evolutionary origin of HIPVs (Allison & Hare 2009; van der Meijden & Klinkhamer 2000; Heil 2008; Janssen *et al.* 2002).

General theory predicts that the evolution of chemical release precedes the evolution of perception, and that chemicals of biological origin may first have different roles before being adopted as infochemicals (Steiger *et al.* 2011). Examples for this concept include cuticular hydrocarbons of insects, which primarily function as desiccation barriers and have been adopted as social signals (Howard & Blomquist 2005) and volatiles that help parasitic wasps to defend themselves against insect predators and have subsequently become an important part of their pheromone blend (Weiss *et al.* 2013). In plants, fragments of cell walls and metabolic enzymes, both of which are important to maintain primary functions, are also used to detect cell damage (Schmelz *et al.* 2007; Heil 2009), and several plant toxins seem to possess signalling-like functions (Meihls *et al.* 2013; Maag *et al.* 2015). As most HIPVs are produced by dedicated biosynthetic pathways and are therefore subject to natural selection (Gershenzon & Dudareva 2007; Frey *et al.* 2000; D'Auria *et al.* 2002), it is conceivable that they may have evolved in a similar manner (Janssen *et al.* 2002; Allison & Hare 2009).

Several recent studies indicate that HIPVs may have direct benefits for the emitter (Maag *et al.* 2015; Allison & Hare 2009). The nitriles benzyl cyanide, 2-methylbutyronitrile, and 3-methylbutyronitrile that are specifically emitted from black poplar (*Populus nigra*) leaves under attack by the gypsy moth (*Lymantria dispar*), for instance, are strongly avoided by the caterpillars and can even paralyze them (Irmisch *et al.* 2014), while their aldoxime precursors are highly attractive to the gypsy moth parasitoid *Glyptapanteles liparidis* (Clavijo McCormick *et al.* 2014). Also, high doses of green leaf volatiles (GLVs), which are rapidly released from wounded leaves through the enzymatic breakdown of membrane lipids, reduce *Spodoptera littoralis* growth (von Mérey *et al.* 2013). Similarly, silencing a hydroperoxide lyase involved in GLV formation in potato increases aphid performance (Vancanneyt *et al.* 2001). Recently, the green leaf volatile (*Z*)-3-hexenol was found to be converted to (*Z*)-3-hexenyl-vicianoside in tomato (*Solanum lycopersicum*), which reduces the survival and growth of *Spodoptera littura* by 10 % (Sugimoto *et al.* 2014). Interestingly, (*Z*)-3-hexenol is also known for its ability to prime defences in maize plants (Engelberth *et al.* 2004). The interpretation of GLVs in the context of HIPVs is complicated by the facts that their release is generally not herbivore specific and does not require the activation of defensive processes (Scala *et al.* 2013). Apart from their potential function in direct defence against herbivores, HIPVs have also been proposed to alleviate abiotic stress that accompanies herbivore attack (Allison & Hare 2009) and to protect wounds against pathogens (Scala *et al.* 2013; Quintana-Rodriguez *et al.* 2015), both of which may provide direct fitness benefits to herbivore-attacked plants without the need of HIPV perception by another organism. The sesquiterpene (*E*)-(β)-caryophyllene, for instance, reduces flower colonization by the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (Huang *et al.* 2012) and can also be used as a cue by root herbivores and their natural enemies (Rasmann *et al.* 2005; Robert, *et al.* 2013). While evidence for direct benefits of HIPVs is accumulating, clear examples of individual herbivore-specific compounds that act as both direct defences and signals within the same plant species are still surprisingly rare.

So far, studies on direct effects of HIPVs on herbivores have used herbivore growth as a proxy for their defensive function (von Mérey *et al.* 2013; Sugimoto *et al.* 2014; Irmisch *et al.* 2014; Li *et al.* 2000). Although herbivore growth can indeed be positively correlated with herbivore fitness and plant damage (Qi *et al.* 2011; Agrawal 1999), several exceptions exist (Lu *et al.* 2015). Low food quality for instance

85 can lead to compensatory feeding, resulting in both higher plant damage and slower herbivore growth
86 (Raubenheimer & Simpson 1999). Also, a surplus of carbohydrates can lead to increased lipid storage
87 (i.e. obesity) and reduced fitness in insects (Warbrick-Smith *et al.* 2006). Therefore, the question
88 whether HIPVs can provide direct benefits to the emitting plant, either by decreasing plant damage or
89 herbivore fitness, remains open. By consequence, it is unclear whether the initial step in the evolution
90 of HIPVs can be explained by current theory (Steiger *et al.* 2011) or whether alternative hypotheses
91 need to be considered (Janssen *et al.* 2002; Allison & Hare 2009).

92 Here, we investigated potential direct benefits for plants to release the HIPV indole. Indole is emitted
93 by many plant species after herbivore attack, including maize (*Zea mays*) (Degen *et al.* 2012), gerbera
94 (*Gerbera jamesonii*) (Gols *et al.* 1999), cotton (*Gossypium hirsutum*) (McCall *et al.* 1994), peanut
95 (*Arachis hypogaea*) (Cardoza *et al.* 2003), lima bean (*Phaseolus lunatus*) (Boer *et al.* 2004) and rice
96 (*Oriza sativa*) (Yuan *et al.* 2008). Its release is triggered by the transcriptional upregulation of the indole-
97 3-glycerol-phosphate lyase IGL (Zhuang *et al.* 2012; Frey *et al.* 2000). IGL is rapidly and specifically
98 induced by herbivory and responds strongly to volicitin (N-(17- hydroxylinolenoyl)-L-glutamine) (Frey
99 *et al.* 2004; Frey *et al.* 2000), an elicitor found in the regurgitant of *Spodoptera* larvae (Alborn 1997).
100 Recently, indole was found to be a strong priming signal in maize (Erb *et al.* 2015). Maize plants that
101 were previously exposed to indole exhibited a significantly stronger jasmonate (JA) burst and released
102 higher amounts of terpenoid HIPVs following simulated herbivore attack, and indole deficient igl
103 mutants were incapable to prime their systemic, non-attacked leaves (Erb *et al.* 2015). Because of its
104 herbivore-specific release pattern, its clear genetic component and its documented function as a priming
105 signal, indole may be a promising candidate to explore potential direct benefits of HIPVs. The results
106 presented here demonstrate that volatile indole directly increases the mortality of early instar *S. littoralis*
107 caterpillars and reduces plant damage. This suggests that the release of indole can benefit plants without
108 the need of an infochemical context.

Materials and Methods

Plants and insects

Two maize (*Zea mays* L.) indole mutants and wild type lines in different genetic backgrounds (termed A and B) were used in this study. For a detailed description of these plant genotypes, see Ahmad *et al.* (2011), Erb *et al.* (2015). All maize lines were grown individually in plastic pots (10 cm high, 4 cm diameter) with commercial potting soil (Aussaaterde, Ricoter, Aarberg, Switzerland) and placed in a climate chamber (23 °C ± 2 °C, 60 % relative humidity, 16:8 h L/D, 50'000 lm/m^2). Maize plants used for the experiments were ten to twelve days old and had three fully developed leaves. The evening before the experiments, plants were transferred and kept under laboratory conditions (25 ± 2 °C, 40 ± 10% relative humidity, 16 h light/8h dark, and 8000 lm/m^2). *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caterpillars were reared from eggs provided by Syngenta (Stein, Switzerland). The eggs were kept in an incubator at 30.0 ± 0.5 °C until emergence of the larvae. Subsequently, they were transferred to artificial diet (Beet Armyworm Diet, Bio-Serv, Flemington, NJ) and kept at room temperature (24 ± 4°C) until the start of the experiments.

Caterpillar performance

To determine whether indole can protect maize plants against *S. littoralis*, we conducted three experiments. In a first experiment, individual wild type and indole deficient igl mutant plants were infested with 4 first-instar *S. littoralis* larvae or left herbivore-free (n=12). Larvae were prevented from escaping by placing transparent 1.5 L poly-ethylene (PET) bottles with the bottom cut out (30 cm height, cone-shaped, maximum diameter 8 cm) over the leaves. The bottles were placed upside down over the plants and attached to the pots with Parafilm (Erb et al. 2011). After 3 days of feeding, *S. littoralis* survival was scored for each plant, and two surviving caterpillars per plant were randomly selected and left to feed for another 4 days. This reduction in herbivore density was necessary to maintain sufficient leaf biomass for *ad libitum* feeding for the entire duration of the experiment. After 7 days, herbivore weight was determined. Furthermore, we measured the fresh mass of the remaining shoots and compared it to the fresh mass of non-infested plants of the same genotype to estimate biomass removal. In a second trial, *S. littoralis* growth and survival on igl-mutants with or without synthetic indole complementation was measured (n=12). To complement the plants with indole, they were watered with 10 mL of an indole

137 solution (100 µg/mL H_2O) every day during the experiment. Indole is taken up by plants (Schullehner
138 *et al.* 2008) and can diffuse through membranes (Piñero-Fernandez *et al.* 2011), rendering this a suitable
139 approach for indole complementation. Plants were infested with 3 caterpillars, and growth and survival
140 was measured after 7 days of feeding. To separate growth effects of volatile indole from plant-mediated
141 effects, we performed an additional experiment in which the larvae were exposed to synthetic indole
142 while feeding on artificial diet. Neonates, first instar or third instar *S. littoralis* larvae were exposed to
143 volatile indole for 6 h (n=12). For this purpose, we used volatile dispensers that were constructed as
144 previously described and emitted indole at a similar rate as wild type plants (approx. 50 $ng*h^{-1}$, Erb *et*
145 *al.* 2015). Briefly, the dispensers consisted of 2 mL amber glass vials (Supelco, Sigma Aldrich, Buchs,
146 Switzerland) that were supplied with 20 mg of synthetic indole (Sigma Aldrich, Buchs, Switzerland).
147 The vials were closed with open screw caps with rubber septa. The septa were pierced with 1 µl
148 micropipette (Drummond Scientific, Broomall, PA) through which indole diffused at a constant rate.
149 Groups of caterpillars (20 neonates, 10 L1 or 5 L2-L3) were placed on a cube of artificial diet (0.5 cm^3)
150 in individual plastic boxes together with a control or indole-releasing dispenser. Neonates were freshly
151 hatched, while L1 larvae were 24 h old and L2-L3 were 48-72 h old. Larval weight gain, diet
152 consumption and survival were measured after 6 h of indole exposure. Diet consumption was measured
153 by recording the weight of the diet cube before and after the experiment, and average weight differences
154 were calculated for each replicate.

155 **Caterpillar feeding preference**

156 To test whether indole affects *S. littoralis* feeding choice, we performed a leaf disc preference assay.
157 Two leaf-discs (5 mm diameter), one from an induced igl mutant and one from an induced wild type
158 plant, were placed as pairs in Petri dishes (diameter 100 mm). Leaf discs were cut from the non-damaged
159 parts of the second true leaf, which had previously been induced by scratching the leaf over two areas
160 of approximately 1 cm^2 on both sides of the central vein with anatomical forceps (stainless steel, 14.5
161 cm). Then 10 µL of *Spodoptera littoralis* regurgitant was applied over the scratched leaf areas. This
162 treatment triggers the releases of the same HIPVs as caterpillar feeding (Turlings *et al.* 1990; Amo *et*
163 *al.* 2013; Sabelis & Dicke 1987). Regurgitant was collected from fourth instar *S. littoralis* that had been
164 feeding on maize leaves for 24 h and was stored at -80 °C until use. One first instar *S. littoralis* larva

was put in the center of each box and was allowed to feed on the leaf disks for 18 h (n=11). Leaf discs were then scanned and the damaged leaf area was measured in Photoshop PS6. In a second experiment, igl mutants were complemented with indole to directly assess its impact on feeding preference. For this purpose, the leaf discs were either submerged in H_2O or an indole solution (100 µg/mL H_2O) for 8 h before the start of the experiment (n=11). In a separate experiment, we measured the solution uptake of leaf discs over 8 h (n=5). On average, a leaf disc absorbed 2.4 (±0.16) µl of solution, which is equivalent to 240 ng of indole and corresponds to a physiologically realistic amount (Degen *et al.* 2012; Glauser *et al.* 2011).

Caterpillar attraction

To test whether *S. littoralis* caterpillars use indole as a host selection cue, we measured the attraction of third instar larvae in a modified four-arm olfactometer (D'Alessandro & Turlings, 2005). The olfactometer consisted of a central glass choice arena (6 cm internal diameter (ID), 5 cm length) with four arms (15 mm ID, 5 cm length), each with a glass elbow (5 cm length) and an upward connection for a glass bulb (50 mL). Purified and humidified air entered each odour source vessel at 1.1 L/min (adjusted by a manifold with four flow meters; Analytical Research System, Gainesville, FL) via Teflon tubing and carried the HIPVs through the connector tube to the elbows of the olfactometer. In these elbows, a part of the air (0.7 L/min) was pulled out and the other part entered in the central glass chamber. Ten neon tubes were attached to a metal frame above the olfactometer and provided approximately 7000 $lm*m^{-2}$ at the height of the odour source vessels. To avoid visual distraction of the larvae, a white cardboard cylinder was placed around the central choice arena and on top of the choice arena. The choice arena was connected to four glass bottles. Two opposite bottles contained an odour source and the two remaining bottles remained empty. The position of the odour sources was changed between each experimental assay. The system was left connected for half an hour before releasing thirty third-instar larvae in the center of the choice arena. The larvae would crawl out of the box and enter one of the four arms. After thirty minutes, larval numbers in each arm were counted. The larvae that did not make the choice arena after thirty minutes were considered as having made “no choice”. Five to six such releases were done for each replicate and pooled for analysis. Using this system, several experiments were carried out. First, we tested the preference of *S. littoralis* for wild type and igl mutant plants (n=5).

To induce HIPV release, all plants were infested with fifteen second-instar *S. littoralis* larvae that were placed on the plant the evening before the bioassay. Second, we assessed the preference of the larvae for *S. littoralis* infested IGL wild type vs. igl mutant plants that were complemented with synthetic indole (n=5). Third, we studied the response of *S. littoralis* to pure indole at 50 and 100 ng*h^{-1} using either a single or two indole dispensers (n=4-5). The chosen release rates cover the natural range of indole emissions measured in different maize genotypes (Erb *et al.*, 2011). For this experiment, we placed indole dispensers in two odor sources and control dispensers in the two remaining sources of the olfactometer.

Oviposition preference

To test if indole affects the oviposition choices in adult *S. littoralis* females, gravid females that had been reared on artificial diet were given a choice between previously infested igl-mutant and wild type plants. For each replicate, eight igl mutant and eight wild type plants (4 of each genetic background) were infested with three *S. littoralis* larvae (L1) per plant to induce indole emission and placed in an oviposition cage (100 cm high, 150 cm long, 50 cm deep). The two mutant lines were placed on one side, and the two wild type lines were placed on the other side of the cage. To prevent the larvae from escaping and the adult moths from directly touching the plants or larvae, a nylon mesh was placed around the plants. Twenty *S. littoralis* pupae (sex ratio=1:1) were then placed in the centre of the cage at equal distance of the infested plants. Seven days later, the eggs laid by the females that had emerged from the pupae on the nylon mesh and cage walls were counted. To assess oviposition preference, the cage was divided into three sections of similar size: The side harbouring the igl mutant plants, the side harboring the wild type plants and the center ("no choice"). Two groups of moths were tested: One group was exposed to volatile indole (50 ng*h^{-1}) during larval development ("experienced", n=4), whereas the other group was exposed to control dispensers ("naïve", n=4). For this purpose, individual *S. littoralis* larvae were reared to pupation in small plastic cages (3 cm x 3 cm x 1 cm) containing a 1 cm^3 cube of wheat germ-based artificial diet and a control or indole dispenser until pupation.

Statistics

Differences in larval performance were assessed using analysis of variance (ANOVA) performed with Sigma Plot 12.5. Holm-Sidak post hoc tests were used for pairwise comparisons. In case of non-

normality or non-equality of variances (as determined by Shapiro-Wilk and Levene's tests), the data was $\log_{10}+1$, square-root or rank transformed prior to analysis. Whenever the applied transformations did not resolve the problems with variance and normality, non-parametric Kruskal-Wallis H tests were performed. Caterpillar survival was analyzed using Kruskal-Wallis H tests. Whenever more than one caterpillar was used per plant or diet cube, performance was averaged and used as unit of replication. To assess larval feeding and adult oviposition preferences, a normalized choice differential was calculated from each replicate by subtracting the percentage of leaf removal or laid eggs of the control side from the percentage on the treatment side. The differentials were then compared against the null hypothesis (equal preference for both sides, resulting in a differential of 0) using ANOVA with the software package R (version 3.2.1.). Differences between backgrounds were evaluated by ANOVA or Kruskal-Wallis H tests. The functional relationship between *S. littoralis* choice and the different odour sources in the olfactometer assays was analyzed with a generalized linear model with R (GLM, log link-function) followed by ANOVA. To compensate for the overdispersion, we based the models on a quasi-Poisson distribution (Turlings *et al.* 2004).

Results

Indole reduces *S. littoralis* survival and food consumption, but increases caterpillar growth

To test if indole affects *S. littoralis* growth and survival, we conducted three performance experiments. In a first experiment, we found that *S. littoralis* larvae suffered from higher mortality on wild type plants compared to indole-deficient igl mutants (*d.f.*=48, *H*=5.9, *P*=0.015; Fig. 1a). Surprisingly, the higher mortality was associated with an increase in larval weight (*d.f.*=43, *H*=19.2, *P*<0.001; Fig. 1b). Both effects occurred independently of the genetic background of the mutation (survival: *H*=0.2, *P*=0.628; growth: *F*=0.9, *P*=0.334). The differences in survival and growth on the different genotypes could be reversed by complementing the igl mutants with synthetic indole (survival: *d.f.*=24, *H*=9.9, *P*=0.002; growth: *d.f.*=24, *F*=13.9, *P*=0.001; Fig. 1c-d). Caterpillars also consumed significantly less plant material on wild type compared to igl mutant plants (*d.f.*=42, *F*=8.4, *P*=0.006; Fig. 1e). Biomass in the genetic background B was significantly higher than in background A (*F*=8.6, *P*=0.005), but the net effect of the igl mutation was similar in both backgrounds (Interaction: *F*=0.0, *P*=0.949). As indole may be incorporated in defensive metabolites *in planta*, including for instance benzoxazinoids (Köhler *et al.* 2014), we performed an additional experiment in which we measured the impact of synthetic indole on the caterpillars while they were feeding on an artificial diet. We observed a rapid increase in mortality of neonates (*d.f.*=35, *H*=8.5, *P*=0.038) and first instar caterpillars (*H*=7.2, *P*=0.026) 6 h after exposure to indole at physiological levels (Fig. 2a). Indole exposed caterpillars gained marginally more weight (*d.f.*=35, *F*=3.8, *P*=0.059; Fig. 2b), despite reduced food intake (*d.f.*=35, *F*=12.9, *P*<0.001; Fig. 2c). Pairwise comparisons revealed a significant effect of indole on weight gain for first instar larvae (Holm-Sidak post-hoc test, *t*=2.4, *P*=0.020) and a significant impact on diet consumption of neonates (*t*=2.1, *P*=0.041) and first instars (*t*=2.2, *P*=0.034). Together, these experiments demonstrate that indole directly increases caterpillar growth, but reduces caterpillar survival and food consumption.

Indole deters *S. littoralis*

To test whether the presence of indole deters *S. littoralis* caterpillars from feeding, we conducted a series of choice experiments. When given a choice between leaf-disks of wild type and indole-deficient igl mutant plants, *S. littoralis* caterpillars clearly preferred to feed on the mutant plants (*d.f.*=19 *t*=4.3, *P*<0.001; Fig. 3a). The effect was significantly stronger in the genetic background A than background

B (H=6.3; P=0.012). Soaking mutant leaf-disks in an indole solution before the experiment significantly reduced the feeding of the caterpillars compared to mutants soaked in water (*d.f.*=19, t=2.2, P=0.042; Fig. 3b). No significant difference between the two backgrounds was observed in this experiment (*d.f.*=19, F=0.35; P=0.564).

Indole producing plants repel *S. littoralis*

To test whether *S. littoralis* caterpillars avoid indole-producing plants from a distance, we performed experiments using a modified 4-arm olfactometer. *S. littoralis* larvae were significantly more attracted to HIPVs from igl mutants than wild type plants (*d.f.*=16, F=79.3, P<0.001; Fig. 3c) independently of the genetic background (Interaction: F=0.2, P=0.716). This effect could be reversed by adding a dispenser releasing indole at a physiologically relevant rate to the mutant plants (*d.f.*=16, F=0.2; P=0.241; Fig. 3d) independently of the genetic background (Interaction: F=4.0; P=0.06). Significantly fewer caterpillars moved towards dispensers releasing indole than to empty control dispensers at 50 ng*h^{-1} (*d.f.*=6, F=7.6, P=0.032) and 100 ng*h^{-1} (*d.f.*=8, F=22.1; P=0.002). Taken together, these results illustrate that indole repels *S. littoralis* caterpillars from maize plants.

Indole reduces *S. littoralis* oviposition

To test whether the plants' capacity to produce indole also reduces herbivore pressure through reduced oviposition, we carried out oviposition experiments with *S. littoralis* adults. When given a choice between wild type and igl mutant plants, *S. littoralis* females preferred to lay eggs in proximity of the igl mutants (*d.f.*=6, t=9.5, P<0.001; Figure 4). This preference was more pronounced for naïve than for experienced moths (*d.f.*=6, t=2.7; P=0.034).

Discussion

Evolutionary theory predicts that HIPVs may not have evolved as infochemicals in the first place (Steiger *et al.* 2011; Weiss *et al.* 2013). Our experiments demonstrate that indole acts as a potent defense that can directly protect the emitting plant against an herbivore. Plants therefore benefit from releasing this volatile even in the absence of any infochemical function.

We found that indole negatively affected the performance of *S. littoralis*: Larvae feeding on wild type plants consumed less biomass and suffered from a higher mortality than larvae feeding on indole deficient mutants. As indole primes plant defences (Erb *et al.* 2015) and can also serve as a precursor for the biosynthesis of toxic benzoxazinoids (Glauser *et al.* 2011; Köhler *et al.* 2014), this result alone does not allow for conclusions regarding the direct effects of indole on the caterpillars. However, early instar caterpillars feeding on artificial diet that were exposed to synthetic indole also suffered from the same effects, showing that indole by itself has a direct negative impact on the caterpillars. The relative contribution of indole as a direct defence, as a defensive signal (Erb *et al.* 2015), or as a secondary metabolite precursor (Köhler *et al.* 2014) remains to be elucidated. In our experiments, indole reduced food consumption by 40 % *in planta* and by 20% on artificial diet, which may be indicative of the expected cumulative influence of its function as both toxin and defense signal in plants. The increase in mortality of neonate and first instar caterpillars exposed to physiological doses of indole after only 6 hours suggests that the molecule is directly toxic or triggers rapid, detrimental metabolic changes in the insect. Indole is known to inhibit cell division of *Escherichia coli* by acting as an ionophore that conducts ions across lipid membranes (Chimerel *et al.* 2012). Furthermore, it can uncouple oxidative phosphorylation and inhibit electron transport in mammals (Sakai M. *et al.* 1982). Even though both effects have been reported for indole concentrations that are several orders of magnitude higher than the emission rates of plants, it is possible that early instar *S. littoralis* caterpillars are particularly susceptible to exogenous ionophores. Indole can diffuse freely across lipid membranes, and it is conceivable that indole from the environment reaches vital organs of the caterpillars rapidly (Piñero-Fernandez *et al.* 2011). Emitting a bioactive HIPV upon herbivore attack clearly increases plant resistance and therefore directly benefits the emitter. This may also explain why indole is produced by a great number of species

310 across the plant kingdom (Degen *et al.* 2012; Gols *et al.* 1999; McCall *et al.* 1994; Cardoza *et al.* 2003;
311 Boer *et al.* 2004; Yuan *et al.* 2008).

312 Interestingly, indole consistently increased caterpillar growth, despite its negative effect on caterpillar
313 survival and food consumption. Caterpillars are known to increase feeding rates on low protein food
314 sources, which can lead to the accumulation of high lipid reserves and to a reduction in caterpillar fitness
315 (Raubenheimer & Simpson 1999; Warbrick-Smith *et al.* 2006). However, in our experiments, indole
316 decreased food intake, pointing to another mechanism related to a change in food conversion efficiency.
317 Food conversion efficiency is defined as mass gain per ingested mass (Scott *et al.* 2010) and depends
318 on a number of factors, including water retention (Timmins *et al.* 1988) and digestive efficiency (Chen
319 *et al.* 2005). Digestive efficiency in insects can be strongly influenced by the gut microbial community
320 (Hansen & Moran 2014). In velvet bean caterpillars (*Anticarsia gemmatalis*) for instance, gut bacteria
321 contribute significantly to protein digestion (Visôtto *et al.* 2009). Indole is a well-known bacterial signal
322 that interferes with quorum sensing (Kim & Park 2013), biofilm formation (Hu *et al.* 2010) and bacterial
323 cell division (Chimerel *et al.* 2012). It is therefore possible that indole exposure alters the gut microbial
324 community of caterpillars in a way that changes their capacity to digest plant material. Further
325 experiments will be needed to test this hypothesis. Caterpillar weight gain is often used as a proxy for
326 plant resistance and as an indicator of the plant's defensive status (Skibbe *et al.* 2008; Rasmann *et al.*
327 2012; Bruessow *et al.* 2010). Our own work is no exception to this (Erb *et al.* 2011; Machado *et al.*
328 2015). The data presented here demonstrate however that measuring weight gain in the context of
329 bioactive secondary metabolites may be misleading, as it may be inversely correlated with biomass
330 consumption and caterpillar mortality.

331 The toxicity of indole coincides with a repellent effect of indole-producing plants on *S. littoralis* larvae
332 and adults. Caterpillars consistently oriented themselves towards plants that did not emit indole and also
333 preferred food sources without indole. Similarly, adult moths preferred to lay eggs on igl mutant over
334 wild type plants. Even though we did observe a slight shift towards indole containing plants by adults
335 that had experienced indole as larvae, the overall choice pattern was similar between naïve and
336 experienced individuals. Similar patterns have been observed in other systems (Akhtar & Isman 2003),

337 and the results confirm the notion that HIPVs play an important role in host plant selection by moths
338 (De Moraes et al., 2001).

339 Although most responses where highly conserved between the two plant genetic backgrounds, *S.*
340 *littoralis* larvae grew better on plants and showed a weaker feeding preference for the igl mutant in the
341 genetic background B. This suggests that non-volatile feeding cues may influence the response of the
342 larvae to indole. From the plant's perspective, the strong avoidance behaviour of the caterpillars may
343 increase the benefit of indole emission and may have favoured that the trait be maintained in natural
344 populations. From the herbivore's perspective, these results suggest that *S. littoralis* has evolved the
345 capacity to perceive the volatile and use it as a cue to avoid toxic plants. HIPVs are used as foraging
346 cues by diverse herbivores and can act as both attractants (Carroll *et al.* 2008; Halitschke *et al.* 2008)
347 and repellents (Clavijo McCormick *et al.* 2014; Bernasconi *et al.* 1998). The direction of the effect may
348 depend on the relative benefits of locating host plants (von Mérey *et al.* 2013), avoiding plants with
349 activated defences (von Mérey *et al.* 2013), escaping from competition (De Moraes *et al.* 2001) and
350 reducing the risk of predation (Grostal and Dicke, 1999). For *S. littoralis*, a generalist that feeds on
351 various indole-producing plants in nature, it seems likely that the benefit of avoiding indole-emitting
352 hosts outweighs the advantage of improved host location. *S. littoralis* has previously been shown to
353 avoid cotton plants of lower quality (Anderson *et al.* 2011), and possesses neurons that respond
354 specifically to indole (Jonsson & Anderson 1999). It remains to be investigated whether indole has
355 similar behavioural effects on other herbivores in order to determine the extent to which indole emission
356 indirectly benefits plants in an infochemical network context (Dicke & Baldwin 2010).

357 Another interesting aspect would be to investigate the role of indole in egg-induced plant responses. Our
358 previous work on a maize hybrid shows that caterpillar-induced indole emission is reduced by
359 *Spodoptera frugiperda* egg deposition (Peñaflor *et al.* 2011), and it is possible that the caterpillars
360 directly benefit from this suppression. On the other hand, certain maize landraces have been shown to
361 produce volatiles in response to egg deposition (Tamiru *et al.* 2011). The role of indole in this context
362 remains to be determined.

From an evolutionary point of view, our findings support current models on the origins of chemical communication. These models assume that HIPVs initially evolved independently of their role as infochemicals (Janssen *et al.* 2002) and that they were subsequently exploited by other organisms in the form of cues, which by definition require no direct communication intent by the emitter (Steiger *et al.* 2011). In a third step, the model predicts that indirect benefits for the emitter derived from the response of the receiver should result in the evolution of HIPVs towards communication signals (Steiger *et al.* 2011). In a previous study, we demonstrated that volatile indole is required for within-plant priming in maize, implying that the HIPV acts as a within-plant signal. Here, we show that indole can directly benefit the emitter by acting as an inducible toxin against a chewing herbivore, and that it is used as a cue by the herbivore to avoid the emitter. These findings demonstrate a direct positive effect on plant resistance of indole, allowing us to propose an evolutionary scenario in which inducible indole emission evolved through duplication and neofunctionalization of a tryptophane synthase alpha subunit (TSA) (Zhuang *et al.* 2012; Frey *et al.* 2009). In a second step, indole was likely adopted as a foraging cue by herbivores like *Spodoptera littoralis* (Jonsson & Anderson 1999). Under the assumption that reducing additional attacks increases plant fitness, the repellent effect may have further stabilized indole emission patterns. As manipulation of a receiver is a form of communication (Rendall *et al.* 2009), indole may already have functioned as an inter-specific signal at this point. At the same time indole likely also evolved into an important within-plant signal (Erb *et al.* 2015), which, together with its role as a repellent, may have consolidated its role as an effective communication signal in maize (Steiger *et al.* 2011). It is important to note that at this point, the proposed evolutionary trajectory is hypothetical, and alternative evolutionary scenarios are certainly conceivable. Depending on the mechanism of indole perception, it is, for instance, possible that the physiological basis to respond to indole was already present when plants evolved the capacity to produce the compound. Indole is a common bacterial volatile, and research on *Arabidopsis thaliana* shows that indole-producing soil bacteria can promote plant growth by modulating auxin signalling (Bailly *et al.*, 2014). Future phylogenetic studies will help to test whether indole release preceded indole perception or *vice versa*.

In conclusion, HIPVs have been shown to function as direct defences, cues and signals (Heil 2014; Allison & Hare 2009; Scala *et al.* 2013), but it had not yet been tested whether individual volatiles if

391 can fulfil all three roles. Our experiments with indole contribute to filling this gap. Our results set the
392 stage for an evolutionary scenario in which a primary benefit allowed for the evolution of a HIPV, which
393 was subsequently followed by its adaptive use as an infochemical by multiple trophic levels.

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Data Accessibility

All data of this manuscript can be found in Appendix S1 in Supporting Information.

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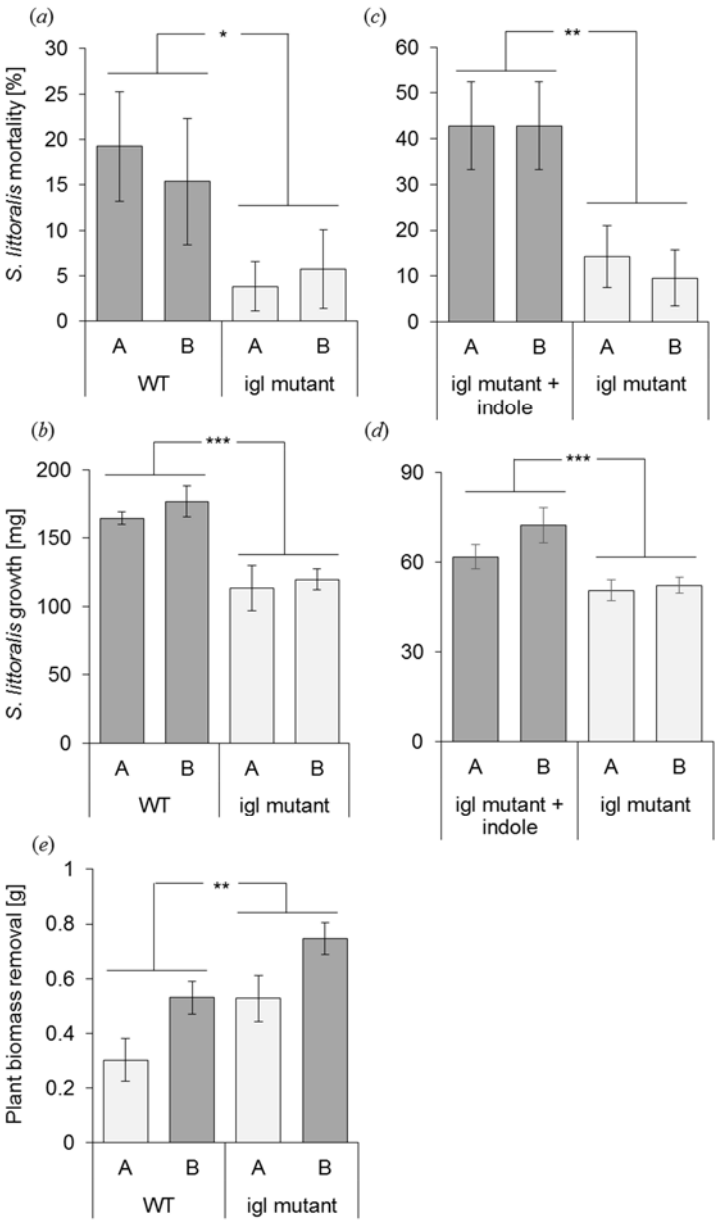
635

Supporting Information

Additional supporting information may be found in the online version of this article:

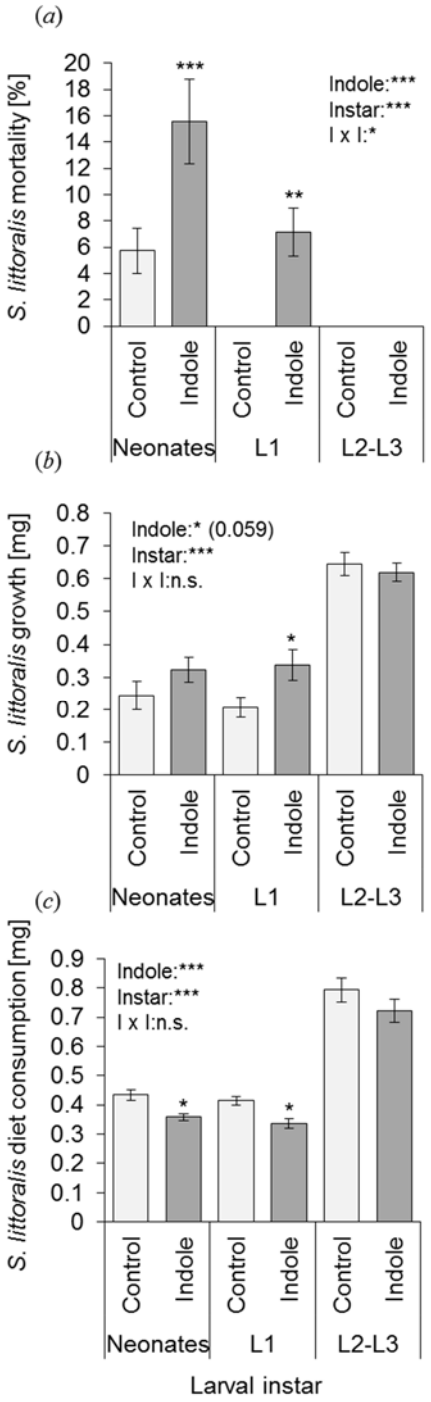
Appendix S1 *Raw data.*

641 *Figures*



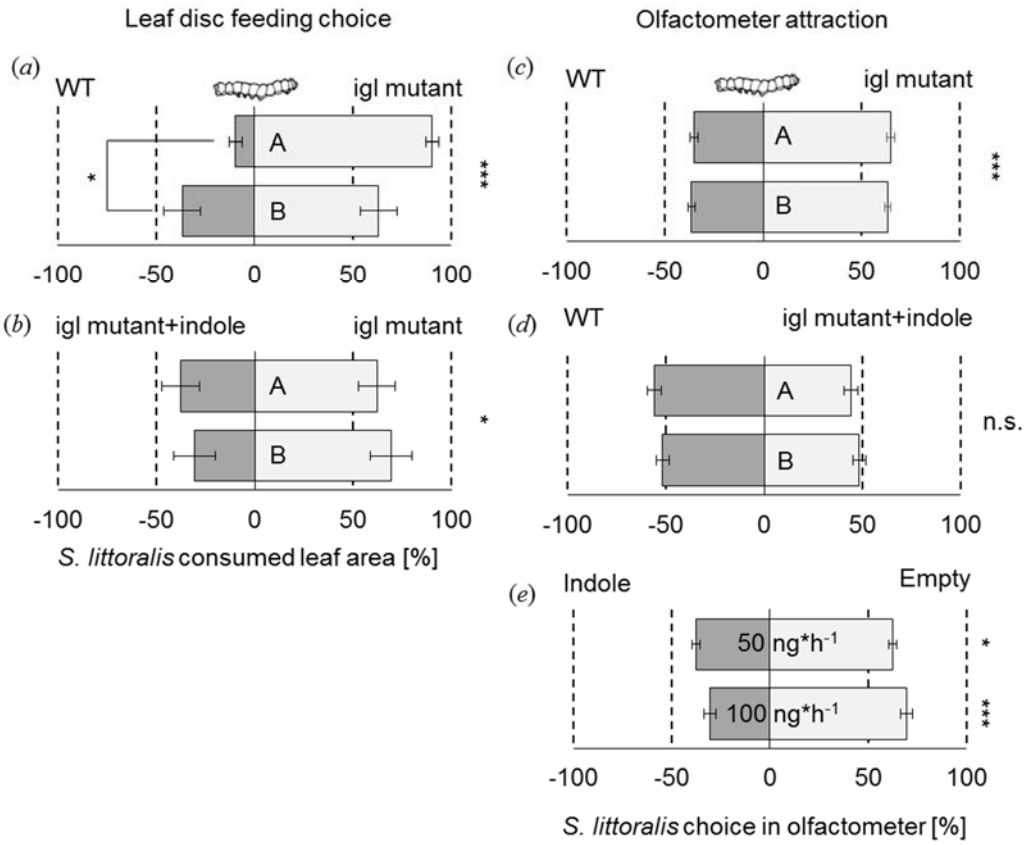
642

643 **Fig. 1.** Endogenous indole increases *S. littoralis* mortality. Growth, survival and plant biomass removal
644 of caterpillars was measured 7 days post infestation on wild type and igl-mutant plants and on igl mutants
645 that were supplemented with indole or not. Stars indicate significant differences between wild type and
646 mutant plants (a, b, e) or between control and indole complemented mutants (c, d). No statistically
647 significant differences were found between genetic backgrounds for a-d. Backgrounds A and B were
648 significantly different from each other in *e* ($p=0.005$). Stars indicate significant differences between wild
649 type and mutant plants (two-way ANOVAs, *$p<0.05$, **$p<0.01$, ***$p<0.001$). Error bars correspond to
650 standard errors (± SE).



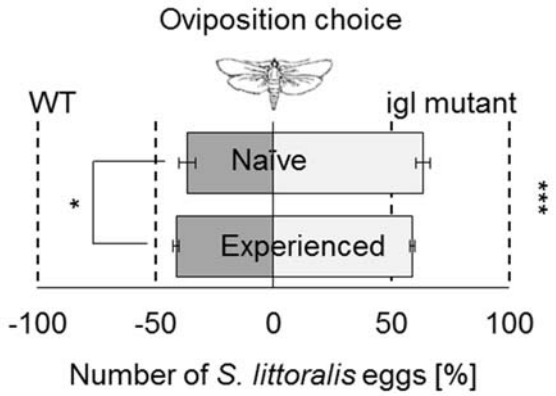
651

652 **Fig. 2.** Synthetic indole increases *S. littoralis* mortality. Different instars of *S. littoralis* caterpillars were
653 exposed to volatile indole (50 ng*h^{-1}) for 6 h, and their survival, growth and artificial diet consumption
654 was recorded over this period of time. Stars indicate significant differences between treatments within
655 instars. *P<0.05, **p<0.01, ***p<0.001. Levels of significance are given for two-way ANOVAs using
656 treatment and instar as factors. Error bars correspond to standard errors (± SE).



657

658 **Fig. 3.** Indole repels *Spodoptera littoralis* caterpillars. (a-b): Choice tests using leaf-disks of herbivory-
659 induced wild type (WT), indole deficient mutant seedlings (igl). For indole complementation, igl leaf
660 disks were soaked in an indole solution (100 µl*mL^{-1} H_2O) prior to the experiment. (c-d): Choice tests
661 in a 4-arm olfactometer using induced seedlings of the different genotypes as odour sources. Indole
662 emission was complemented using capillary dispensers with a release rate of 50 ng*h^{-1}. (e) Choice tests
663 in a 4 arm olfactometer using synthetic indole at different release rates. Stars indicate significant
664 differences between leaf disks, odor sources or genetic backgrounds (ANOVA, *p<0.05, **p<0.01,
665 ***p<0.001). A and B denote the two different genetic backgrounds that were used in the experiments.
666 Error bars correspond to standard errors (± SE).



667

668 **Fig. 4.** Indole reduces oviposition by *S. littoralis* females. Gravid females were allowed to oviposit in a
669 nylon mesh cage with WT plants on one side and igl mutant plants on the other side. Adults from naive
670 caterpillars reared on artificial diet and experienced caterpillars reared on artificial diet supplemented
671 with indole were tested separately. Stars indicate significant differences between plant genotypes.
672 **$p<0.01$. Error bars correspond to standard errors (± SE).